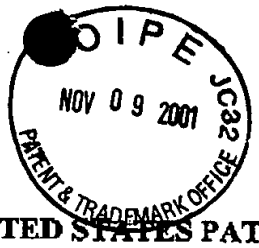
#15



Express Mail Label No.: EL326923155US
Date of Deposit: November 9, 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Carstens, Carsten-Peter		
Serial No.:	09/492,590	Examiner:	G. Leffers Jr.
Filed:	January 27, 2000	Group:	1636
Entitled:	METHODS AND COMPOSITIONS FOR HIGH LEVEL EXPRESSION OF A HETEROLOGOUS PROTEIN WITH POOR CODON USAGE		

Attorney Docket No.: 25436/1340 (Formerly 4114/85530)

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. §1.131

I declare:

1. I, Carsten-Peter Carstens, am the inventor of the invention claimed in the above-noted U.S. patent application.

2. I have read and understood the Office Action mailed May 9, 2001, and have read and understood the cited reference, U.S. Patent No. 6,214,602 ("the '602 patent," issued to Zdanovsky et al. on April 10, 2001, from an application filed August 28, 1998). I understand that the Examiner has cited the '602 patent as a novelty reference over claims 1-10, 15, 16, 19, 22-27, 32-40 and 42-44, and as an obviousness reference over claims 18, 20 and 21. The '602 patent is cited as teaching vectors and methods for the overproduction of Clostridium toxins and proteins by hosts such as E. coli. The methods taught by the '602 patent are said to feature the use of host cells containing a recombinant expression vector, wherein the expression vector encodes tRNAs that recognize rare codons and wherein the host cell expresses at least a fragment of at least one clostridial protein. The patent is also said to teach vector constructs that encode three tRNAs that recognize rarely used codons.

3. Prior to the August 28, 1998 filing date of the '602 patent, I had both conceived of and reduced to practice the claimed invention. The attached exhibits A and B, consisting of copies of my notebook entries detail the experiments that gave rise to the claimed invention, all performed before August 28, 1998. The dates on these exhibits have been redacted. For convenience, Appendices I and II contain transcripts of the relevant notebook entries.

A. Vector comprising an array of three or more tRNA genes which correspond to codons that are rarely used in a host cell

The first set of notebook entries, Exhibit A (transcripts in Appendix I), describes the development of pACYC-based vectors encoding genes for three recombinant tRNAs specific for rarely used codons. The first array is termed "RIL," for *ArgU* (R), *IleY* (I) and *LeuW* (L).

First, even the initial entry of Exhibit A (Entry # 1), describing experiments performed before August 28, 1998, shows that the vectors comprising an array of three tRNA genes were conceived of even before that date. That is, because Entry # 1 details efforts to ligate the RIL insert into the pACYC-LIC vector, I already had the critical RIL insert fragment and the intent to put it into an expression vector that would meet the limitations of the claims.

Second, Entry # 6 shows the ligation that generated pACYC-RIL vector constructs 49, 50 and 55, which were shown in the ensuing experiments (e.g., the PCR screen of Entry # 8 and the miniprep digests of Entry # 9) to contain the RIL insert, all performed before August 28, 1998. Thus, the vectors comprising an array of three or more tRNA genes which correspond to rarely used codons as claimed in the present application were reduced to practice as of the date of entry of Entry # 6, which is prior to August 28, 1998. The vectors resulting from the ligation were screened, isolated and confirmed by the date of Entry # 9, which is also before August 28, 1998. While it is clear that the vectors were made as of the date of Entry # 6 but not confirmed until the date of Entry # 9, both experiments were performed in advance of the August 28, 1998 priority date of the '602 patent.

B. Host cell containing a recombinant DNA molecule which comprises an array of three or more tRNA genes, wherein the tRNA genes correspond to codons that are rarely used in the host cell

The intent at the time of making the pACYC-RIL constructs was to introduce them to host cells for their influence on the expression of heterologous genes with rare codons. Thus, the conception of the claimed host cells that comprise an array of three or more tRNA genes, wherein the tRNA genes correspond to codons that are rarely used in the host cell, necessarily preceded, or at a minimum, coincided with, the conception of the vectors themselves. Therefore, Exhibit A and Appendix I are also sufficient to demonstrate conception of the claimed host cells before August 28, 1998.

Similarly, Exhibit A and Appendix I are sufficient to demonstrate reduction to practice of host cells comprising an array of three or more tRNA genes, wherein the tRNA genes correspond to codons that are rarely used in the host cells. Specifically, the transformation of XL1-Blue host cells with the ligation reactions of Entry # 6 created host cells carrying such a construct. The PCR screening documented in Entry # 7 and the miniprep digests of Entry # 9 show that the pACYC-RIL clones 49, 50 and 55 are positive for the RIL insert. Finally, the test of Flp expression documented in Entries 10, 11 and 12 shows the introduction and induction of the pACYC-RIL construct in the well-

known protein expression E. coli host strain BL21(DE3). Each of these experiments was carried out before August 28, 1998.

In view of the above, host cells as claimed were conceived of and reduced to practice in advance of the August 28, 1998 priority date of the '602 patent.

C. Method of producing a protein of interest

The second set of notebook entries, Exhibit B (transcript in Appendix II), describes the use of the pACYC-RIL constructs to increase the production of a protein of interest from a gene with a non-*E. coli* codon bias.

The conception of a method of producing a protein from a gene with infrequently used codons, the method involving the expression of specific recombinant tRNAs from a vector in a host cell is necessarily very closely linked in time, if not coincident with or even prior to, the conception of the vector and host. Thus, conception of the method occurred before the performing the experiments described in the earliest entry in Exhibit A, which is prior to August 28, 1998. The reduction to practice is described below.

The hyperthermophilic archaeon *Pyrococcus furiosus* has a very AT-rich genome that results in distinctly different codon usage from the less AT-rich *E. coli* host cell genome. Therefore, one wishing to express *Pfu* DNA polymerase in *E. coli* will achieve only limited expression unless codon bias is compensated.

Exhibit B notebook Entry # 1 (page 48) shows the transformation of BL21(DE3)-RIL cells (corresponds to isolate 49) with a *Pfu* polymerase fusion protein. Entry # 2 shows a Coomassie-stained gel containing host cell proteins from *Pfu* polymerase construct-transformed cells with and without IPTG induction of tRNA construct expression. The gel photo shows the production of the *Pfu* polymerase fusion protein. The gel also shows, and the notebook entry states, that the production of the *Pfu* polymerase (rich in rare arg codons and Ile codons), is enhanced in RIL 49 host cells. Both experiments have been performed before August 28, 1998.

Exhibit B Entry # 2 therefore shows reduction to practice for a method of producing a protein of interest, comprising the step of culturing a host cell containing a recombinant DNA molecule that comprises an array of three or more tRNA genes, wherein the tRNA genes correspond to codons that are rarely used in the host cell, wherein the codons are present in the gene for the protein of interest, and wherein the conditions of culturing the host cell are sufficient to produce the protein of interest. The reduction to practice thus occurred prior to August 28, 1998.

4. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

11/18/01
Date


Carsten-Peter Carstens

Appendix I
Transcripts of notebook entries relating to vectors and host cells

(Note -- several unrelated constructs were also being made in addition to the tRNA array constructs):

Entry #1

[page 066]

annealing of LIC-cloning reactions

1 µl of LIC treated vector (pACYC-LIC or pESP4)
5 µl LIC-treated inserts (Flp, Ku70 and Ku80 and RIL)
4 µl dH2O
incubated for 1 h at RT

vectors setup: pESP4Ku70, pESP4Ku80, pESP4Flp and pACYC-Flp and RIL

used 1 µl to transform XL1-Blue cells. Plated transformations on LB-Amp (pESP4 clones) or LB-Cam plates (pACYC based).

Entry #2

[page 067]

PCR Screening of pACYCFlp, pACYC-RIL, pESP4-Ku70, pESP4-Ku80 and pESP4-Flp colonies (Continuation of previous page)
[details PCR reaction components and cycling conditions]

[bottom of page 067, below gel photo]
None of the pACYC-RIL screened are positive.

Entry #3

[page 068]

Screening of more pACYC RIL clones (cont' from prev. page)
[details reaction setup and cycling conditions and shows diagnostic gel; no positive clones detected]

Confirmation diogests of RIL fragment used for LIC cloning of pACYC-RIL and miniprep digest of selected ESP4Flp, ESP4 Ku80 and ESP4 Ku70 clones

[bottom of page, next to lower gel photo]
test of RIL fragment

undigested:	610 bp
NcoI:	220 bp, 390 bp (high salt retards small frag. – NcoI uses 2X UB (*universal buffer*))
XbaI:	180, 430 bp (+LIC sites)

the RIL fragment seems to be correct
unclear why I can't clone – toxic?

Entry #4

[page 080]

Ordered new primer for construction of RIL array. LIC cloning appeared to be very ineffective in previous attempts. Try to clone between the SpeI and XhoI sites of pACYC-LIC. Also ordered forward and reverse primer for RI and IL array to be cloned the same way.

Entry #5

[page 084]

PCR-amplification of RIL, IL and RI fragments using primer with Spe/Xho extensions
[details reaction setup and cycling conditions and isolation of amplified fragments following gel electrophoresis]

Entry #6

[page 086]

Construction of pACYC-RIL, pACYC-RI and pACYC-IL.

a) vector digest *[details digest of pACYC-LIC with SpeI and XhoI]*

b) ligation *[details ligation using 2 µl of cut pACYC-LIC vector and 5 ml of RIL, RI or IL]*

used 2 µl to transform XL1 Blue

Entry #7

[page 087]

PCR screen of pACYC-RIL and pACYC-IL (see prev. page)
[details reaction setup and cycling conditions and shows gel photo]

expected sizes:
RIL 610 bp
IL 430 bp

pACYC-RIL: clone #7
pACYC-RI: 23, 25, 28, 29, 32, 33

Entry #8

[page 090]

minipreps of pACYC-RI and pACYC-RIL colonies (continuation of page 87)

expected fragment sizes
2192, 1230 (RI) or 1400 (RIL)

both pACYC-RI clones are confirmed by restriction digests.

None of the RIL clones is positive although they were PCR positive (comp. page 87)

[page 091]

Rescreened pACYC RIL colonies by PCR
[details reaction setup and cycling conditions, shows gel photo]

none of the previous picked colonies are positive.

3 of the new colonies (49, 50 and 55) are positive.

Entry #9

[page 091]

minipreps of pACYC-RIL clones (above)
expected
NcoI: 2192 bp, 1400 bp
XbaI:

All clones are positive by NcoI digests, but XbaI site seems to be missing (either from vector or from insert).

Entry #10

[page 095]

test of Flp expression in pACYC-RIL/BL21DE3 cells
picked colonies from transformations on page 93 (one each expression clone in each RIL strain) and inoculated O/N cultures (1 ml Amp/Cam LB)

Entry #11

[page 095]

induction of Flp expression in BL21DE3/RIL strains
[details growth and induction of expression of Flp]

Entry #12

[page 096]

detection of Flp expression in BL21DE3 RIL strains
[details gradient gel electrophoresis and Western blotting of samples from cells carrying RIL 49, RIL 50 and RIL 55 constructs]

Signal extremely strong. Had to add piece of paper between the membrane and the film to achieve reasonable exposure times (chemiluminogram next page). Took image with Eagle eye (signal strong enough for that).

Appendix II

Transcripts of notebook entries relating to production of a protein of interest

Entry #1

[page 48]

Comp IRL cells

[details making BL21DE3-RIL cells competent for transformation]

transformed 100 µl ea. with

Pfu polymerase (CBP fusion)	} archaeal protein
Flavo PEF	} AT rich
CreProL#5	

plated on LB Amp.

Entry #2

[page 55]

Coomassie gel of NusA production and RIL test.

production of Pfu-polymerase

(rich in rare arg codons and IleY codons) is enhanced in RIL49. At least the argU component of the strain enhances Pfu production.

inoculated clones BcAmp for #5, 6 and 4 for minipreps

inoculated o/n culture of BL21 DE3 RIL for comp cells

comp RIL cells

inoculated 10 ml LB with 100 µl o/n culture

↓

let grow for 3h at 37°C in shaker

↓

pelleted cells for 15' at 2500 rpm in tabletops

↓

resuspended in 2.5 ml FSB

↓

pelleted 15 at 2500 rpm

↓

resuspended in 600 µl FSB

↓

transformed 100 µl ea. with

Pfu Polymerase (CBD fusion) } archaeal protein
Flavo PEF } At rich
CreProL#5

plated on LB Amp

Confirmation PCR of clones 56 and 11 (miniprep DNA)

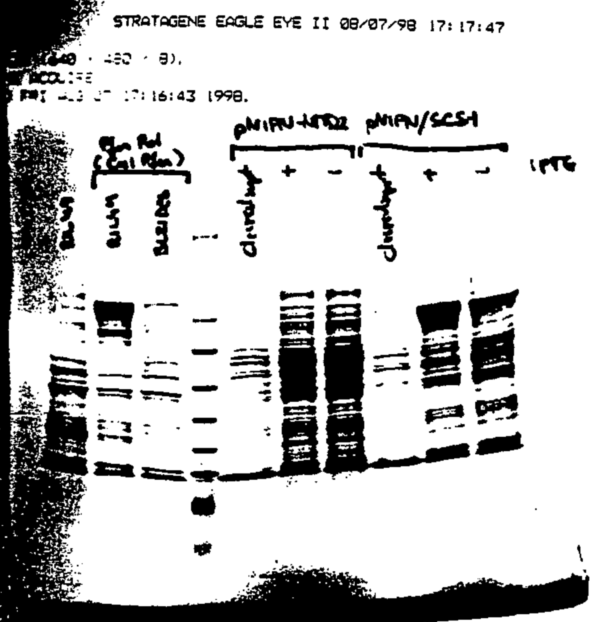
	A	B	C	
10x Taq poly	30	30	~~30~~	95°C, 3' / 55°C, 3' / 72°C, ~~3~~' / 1 cycle
10x cloned Pfu b.	~~[illegible]~~	~~[illegible]~~	30	95°C, 1' / 55°C 1' / 72°, 2' // 24 cycles
dNTPs (25 mM ea)	2.4	2.4	2.4	72°C, 10' → 6°C, hold
1047	.5	–	–	
1048	–	.5	–	loaded 5 µl each sample on
51C	.5	.5	–	1% agarose gel
53C	–	–	.5	
54C	–	–	.5	image next page
PEF	–	–	1	
Taq 2000	2	2	–	

C. C. O.

[illegible]

transformed BL21 gold with 1μl of pNIFN (SCS-1 prep)

no colonies from BL21 transformation with pNIFN



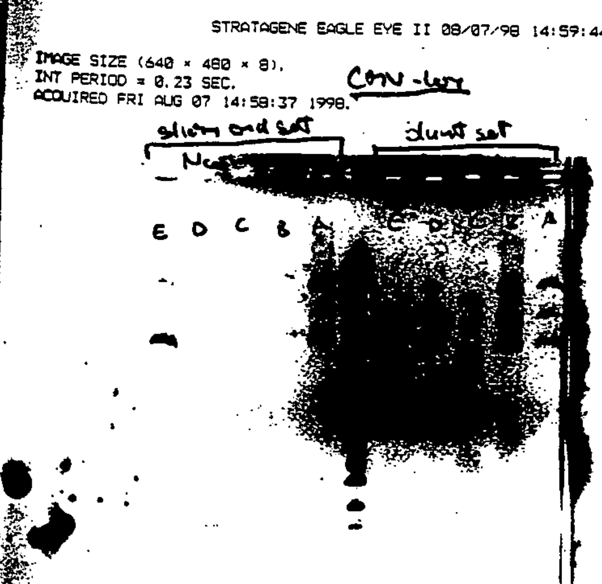
Coomassie gel of NusA production and RIL test.

production of Pfu-polymerase (rich in rare arg codons [illegible] codons) is enhanced in [illegible] → at least the arg U component of the strain enhances Pfu production.

NusA / IFN γ production

No NusA / IFN γ seen in NM522

IFN γ / NusA appears to be strongly produced in SCS-1 cells, even in the absence of induction, but the protein appears to be insoluble



minipreps of CMV-lux ligations (pages 50 and 52)

Expected: 2043, 1600

poor quality minipreps.
very variable yields, appear to be inconsistent

EXHIBIT A

066

ENTRY # 1

annealing of LIC-cloning reactions

1 µl LIC treated vector (pACYC-LIC or ESP4)
5 µl LIC treat. inserts (Flp, Ku20 and Ku80 and RIL)
4 µl ddH_2O
incubated for 1h at RT

Vectors set up: pESP4 Ku20, pESP4 Ku80, pESP4 Flp and pACYC-Flp and RIL

goal of pACYC-Flp → Flp activity is easier to detect in an in vitro assay → quality control of the insert

used 1 µl to transform XL1-blue cells. plated transformations on LB Amp (ESP4 clones) or LB Cam plates (pACYC based)

PCR amplification of kanamycin resistance marker from pACYC-177 for complementation of orc-deleted ComP strain

	1	2
10x Pfu pol buffer (colored)	10	10
dNTPs (25 mM)	.8	.8
PEP	1	1
pACYC-177	1	1
CC 1830 (kan fwd + 2C2)	.5	–
CC 1824 (kan fwd)	–	.5
CC 1156 (kan rev.)	.5	.5
cloned Pfu Pol	2	2
ddH_2O	84.2	84.2

used hot top
95°C, 3' / 55°C, 3' / 72°C, 3' // 1 cycle
95°C, 1' / 55°C, 1' / 72°C, 3' // 24 cycles
72°C, 10' → 6°C, ∞

loaded reactions on 1% agarose gel

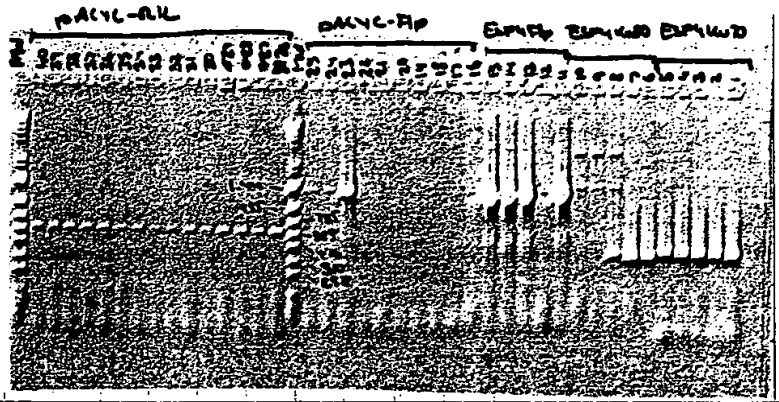
Expected product size: 1.145 Isolated fragments using Strataprep gel extraction kit
eluted DNA into 50 µl TE

PCR screening of pACYC Flp, pACYC-RIL, pESP4-ku70, pESP4-ku80 and pESP4-Flp colonies (Continuation of previous page)

	1	2	3	4	
10x Taq poly buffer	25	25	75	100	used 47.5 µl / reaction
dNTPs (25mM ea)	2	2	6	8	
CC 937 (ku70 screen)	.5	-	-	-	95°C, 3' / 55°C, 3' / 72°C, 2' // 1 cycle
CC 938 (ku80 screen)	.5	-	-	-	95°C, 1' / 55°C, 1' / 72°C, 2' // 29 cycles
CC 939 } ku80 screen	-	.5	-	-	72°C, 10' → hold at 6°C
CC 940 }	-	.5	-	-	used hot Taq
CC 1375 }	-	-	.5	-	
CC 1376 } Flp	-	-	.5	-	loaded 10 µl per reaction on
CC 1605 (Cargill Taq)	-	-	-	1	1% agarose gel
CC 1725 (Lucigen)	-	-		1	
~~dd~~ Taq 2000	2.5	2.5	7.5	10	
ddH2O	21.5	21.5	60	80	

STRATAGENE EAGLE EYE II 04/06/98 16:09:59

IMAGE SIZE (640 × 480 × 8).
INT PERIOD = 0.16 SEC.
ACQUIRED MON APR 06 16:08:46 1998.



expected product sizes
ku70:
ku80:
Flp : 1270 bp
RIL : 610 bp

all ku70 clones, the ku80 clones 6 and 7, ESP4 Flp clones 11 and 13-15 and pACYC clone 23 are positive in PCR screen → pooled for subsequent DNA

none of the pACYC-RIL screen are positive

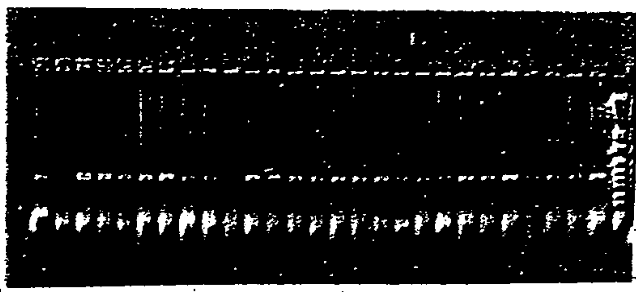
C.C.

Xing Hu

Screening of more pACYC-RIL clones (cont. from prev. page)

STRATAGENE EAGLE EYE II 04/07/98 18:54:46

IMAGE SIZE (640 × 480 × 8).
INT PERIOD = 0.19 SEC.
ACQUIRED TUE APR 07 18:53:02 1998.



10x Taq poly buffer	150
dNTPs (25mM ea.)	12
CC1605	1
CC1725	1
Taq 2000	10
ddH2O	1326

used 47.5 µl ea reaction

95°C, 3' / 55°C, 3' / 72°C, 2 // 1 cycle

95°C, 1' / 55°C, 1' / 72°C, 2' // 29 cycles

72°C, 10' → 6°C, ∞

loaded 10 µl / lane, 1% agarose gel

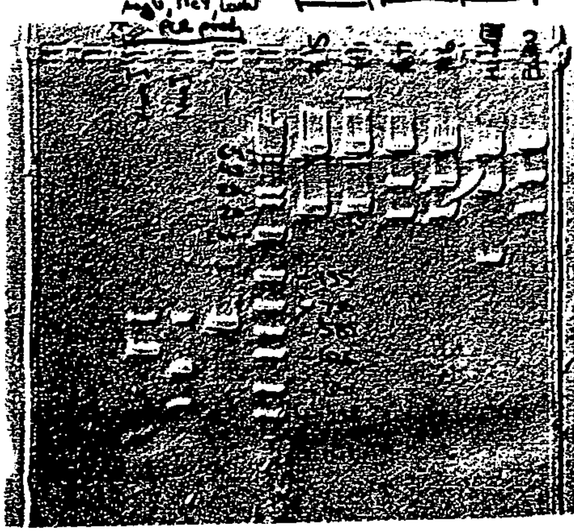
all the bands are too faint for positives

Confirmation digests of RIL fragment used for LIC cloning of pACYC-RIL and miniprep digest of selected ESP4Flp, ESP4Ku80 and ESP4Ku70 clones

STRATAGENE EAGLE EYE II 04/07/98 18:50:02

IMAGE SIZE (640 × 480 × 8).
INT PERIOD = 0.33 SEC.
ACQUIRED TUE APR 07 18:48:13 1998.

Esp4Flp Esp4Ku80 Esp4Ku70
NdeI EcoRV #1
λ/MspI, 1 kb, ladder, RIL prod



expected fragment sizes:

ESP4Ku70 : EcoRV : 7332, 2549, 1660
Hind III : 5841, 2398, 2300, 1022
(not separated)

ESP4Ku80 EcoRV : 7727, 2549, 1660

ESP4Flp NdeI : 9111, 1898

(maps of ESP2Ku70 and ESP2-Ku80 next page)

all digests result in the expected pattern

test of RIL fragment

undigested : 610 bp

NcoI : 220 bp, 390 bp (high salt retards small frag.)

XbaI : 180, 430 bp (+LIC sites)

NcoI cuts 2x US

the RIL fragment seems to be correct

unclear why I can't clone → toxic?

STRATAGENE IN-HOUSE OLIGONUCLEOTIDE REQUEST FORM

REQUESTED BY: Carsten Carstens DATE: 4/22/99

ACCOUNT #'S: (BUSINESS) 22-32650 (PERSONAL) 8122

SEQUENCE(S) 5'→3':

1. ArgU fwd/SpeI

____ 5'- GAG/ACT/AGT/AAT/CAG/ACG/CGG/TCG*
TTC/AC_/___/___/___/___/___/___*
29 bp ___/___/___/___/___/___/___/___ 3'

2. LeuU rev/NheI

____ 5'- GAC/CTC/GAG/GGC/ATC/CGA/TCA/ACG*
CTT/TCT/___/___/___/___/___/___*
30 bp ___/___/___/___/___/___/___/___ 3'

3. IleY rev/NcoI

____ 5'- GAC/CTC/TGA/TCA/TAA/TGT/TTA/TTG*
CGT/GG_/___/___/___/___/___/___*
29 bp ___/___/___/___/___/___/___/___ 3'

4. IleY fwd/SpeI

____ 5'- GAG/ACT/AGT/CCT/TGA/AAT/GGC/GTT*
AGT/CA_/___/___/___/___/___/___*
29 bp ___/___/___/___/___/___/___/___ 3'

5' MODIFICATIONS?: 1.____ 2.____ 3.____ 4.____

PURIFICATION METHOD (circle one for each):

1. Etoh ppt / PAGE 2. Etoh ppt / PAGE 3. Etoh ppt / PAGE 4. Etoh ppt / PAGE

COMMENTS/SPECIAL REQUESTS:

(oligo department use only)

DATE/TIME REC'D: _____ DATE NEEDED: _____

COMPLETED BY: _____ DATE COMPLETED: _____

ordered new primer for construction of RIL array. LIC cloning appeared to be very ineffective in previous attempts.
→ try to clone between the SpeI and NcoI sites of pACYC-LIC

also ordered forward and reverse primer for RI and IL array to be cloned the same way

Test of production of Ku80 and Flp as GST fusion proteins in yeast

goal: produce the said proteins a GST fusion proteins. Previously, failed as CBP fusion proteins either in E.coli or yeast as a host

pulled colonies from SP-Q01 yeast cells transformed with ESP4/Ku80 and ESP4/Flp (pages 68 and 70) on Ura plates → incubated at 30°C

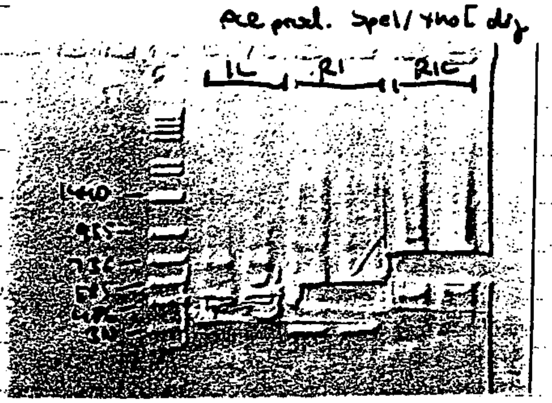
C.C

(continued)
↓
Split each culture into 2 x 5ml cultures
↓
pelleted cells from each 5 ml culture (10' at 2500 rpm, tabletop)
↓
washed 2x with 50ml sterile ddH₂O
↓
resuspended each pellet in 10ml unsupplemented EMM.
Added to one culture each group 10 µl thiamine (5mM).
Becomes "repressed/uninduced" sample
↓
incubated at 30°C in shaker at 250 rpm (o/n incubator)

PCR-amplification of RIL, IL and RI fragments using primer with SpeI/XhoI extensions

GENE EAGLE EYE II 04/30/98 11:38:11
).
:48 1998.

PCR prod. SpeI/XhoI dig



	1	2	3
10x Taq + Prec.	10	10	10
dNTPs	.8	.8	.8
UC-RIL (page 47)	1	1	1
CC 972B (argI fwd)	.5	.5	-
CC 973B (leuU rev.)	.5	-	.5
CC 974B (ileY rev.)	-	.5	-
CC 975B (ileY fwd)	-	-	.5
Taq + Prec.	1	1	1
ddH₂O	86.2	86.2	86.2

95°C, 3' / 55°C, 3' / 72°C, 2' // 1 cycle
95°C, 1' / 55°C, 1' / 72°C, 2' // 19 cycles
72°C, 10' → 6°C hold

isolated PCR products using Strataprep PCR purification kit.
eluted DNA into 50 µl 1x Universal buffer
- added 2 µl SpeI and 2 µl XhoI to each purified reaction
- incubated 2h at 37°C
- loaded on ~~1%~~ 1.5% agarose gel
(continued)

C.O.

(Continued) ↓

1 gel transfered to nitrocellulose membrane by semi-dry transfer

↓

kept in TBS, 5% dry milk at 4°C in cold room

other gel stained with Coomassie

Construction of pACYC-RIC, pACYC-RI and pACYC-IC

a) vector digest
- 10 µl pACYC RIC minimp
- 5 µl 10x U.B.
- 1 µl SpeI
- 1 µl XhoI
- 33 µl ddH_2O

Incubated 1 h at 37°C

removed enzymes with ~~PCR purification~~ buffer (Stratagene PCR purification kit). Purified through spin cups and eluted DNA into 50 µl 20 mM Tris-HCl pH 8.0

b) ligation
- 2 µl vector (pACYC-LIC)
- 5 µl RIC, RI or IC (page 84/85)
- 4 µl 5x ligase buffer
- 1 µl T4 DNA ligase
- 8 µl ddH_2O

incubated for 4 h at 16°C (robocycler cold block)

↓

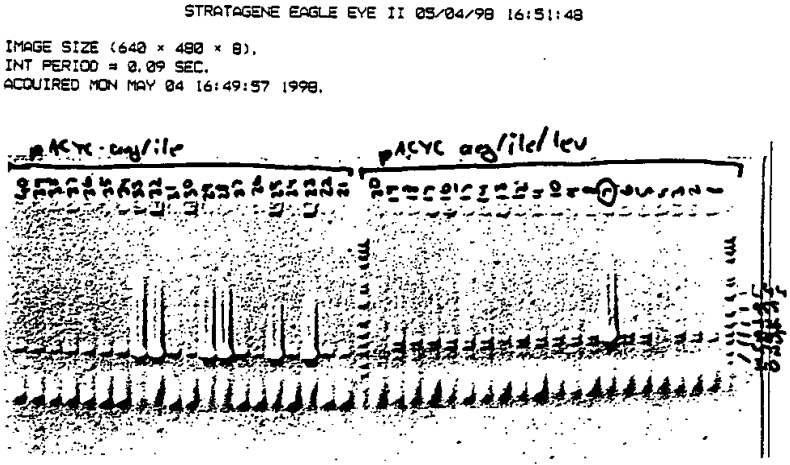
used 2 µl to transform XL1 blue

C. Cato

PCR screen of pACYC-RIL and pACYC-IL (see prev. page)

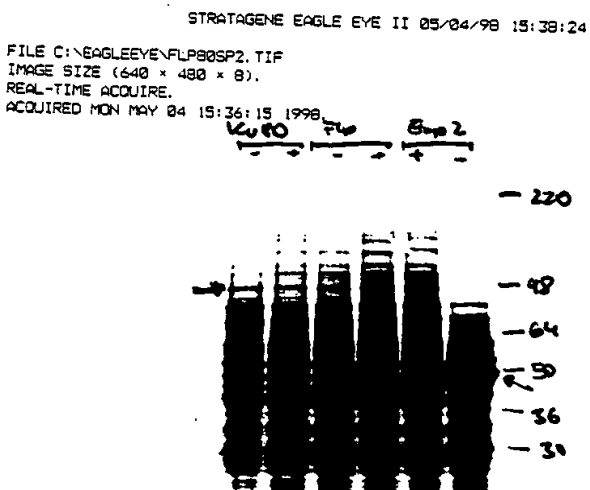
	A	B	
10 x Taq Poly Buffer	100	100	95°C, 3' / 55°C, 3' / 72°C, 2' // 1 cycle
dNTPs (25 mM ea.)	8	8	95°C, 1' / 55°C, 1' / 72°C, 2' // 29 cycles
CC 972 B (arg U fwd)	1	1	72°C, 10' → 6°C, cooled
CC 973 B (Leu W rev.)	1	-	cored out rep
CC 974 B (Ile Y rev.)	-	1	
Taq 2000	10	10	loaded 10 µl ea. reaction on 1.2%
dd H_2O	880	880	agarose gel

Expected sizes:
RIL 610 bp
IL 430 bp



pACYC-RIL : clone #7
pACYCRI : 23, 25, 28, 29, 32, 33

Coomassie gel of induction of P16 and Xu80 in yeast (cont'n. of page 85)



lanes slightly overloaded
only the GST control (E5/2)
has an induced band visible
(arrow)
there may be a very weak
induced band at ≈ 110 kD in
the Xu80 sample, which
would be roughly the
expected size

C.C.

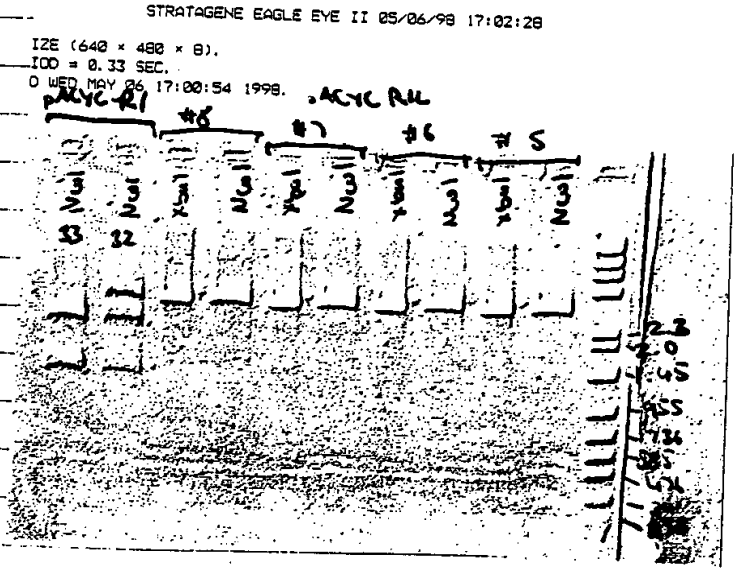
(Continued)

digested vector preps for 2h at 37°C

↓

isolated DNA using Stratuprep PCR purification Cart
(+150 µl DNA binding Soln. → on spin cups)
eluted DNA into 50 µl TE

ligation: 2 µl vector preps (C2 or C6)
5 µl inserts (4.1A or 4.1B)
4 µl 5x ligase buffer
1 µl T4 DNA ligase
8 µl dd H_2O
ligated at 4°C (refrigerator) o/n



minipreps of pACYC-RI and pACYC-RIL colonies (continuation page 87)

expected fragment sizes

2192 bp, 1230 (RI) or 1400 (RIL)

both pACYC-RI clones are confirmed by restriction digests.
None of the RIL clones is positive although they were PCR positive (comp. page 87)

ENTRY # 9

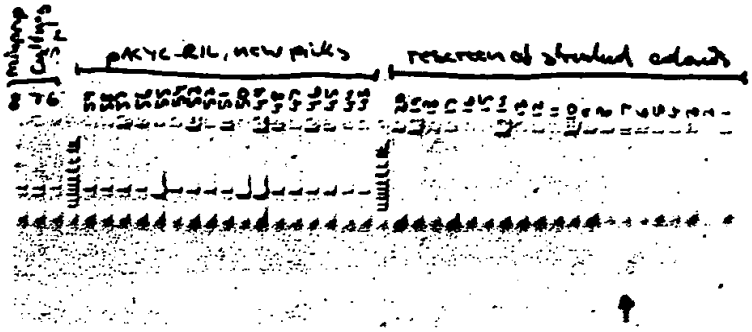
091

Rescreened pACYC-RIL colonies by PCR

10x Taq-polybuffer	200	94°C, 3' / 55°C, 3' / 72°C, 2' // 1 cycle
dNTPs	16	94°C, 1' / 55°C, 1' / 72°C, 2' // 29 cycles
CC 972B	2	72°C, 10' → 6°C, hold
CC 973B	2	used hot top
Taq 2000	10	
ddH2O	1770	loaded 5 ml on 1% agarose gel

STRATAGENE EAGLE EYE II 05/06/98 16:02:19

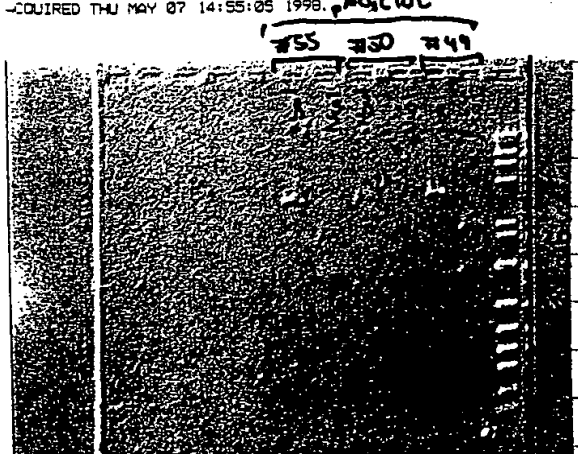
IMAGE SIZE (640 × 480 × 8).
INT PERIOD = 0.16 SEC.
ACQUIRED WED MAY 06 16:00:05 1998.



None of the previous picked colonies were positive.
3 of the new colonies (49, 50 and 55) are positive

STRATAGENE EAGLE EYE II 05/07/98 14:57:3

IMAGE SIZE (640 × 480 × 8).
INT PERIOD = 0.49 SEC.
ACQUIRED THU MAY 07 14:55:05 1998.



Minipreps of pACYC-RIL clones (above)

Expected
NcoI: 2192 bp, 1400 bp
XbaI:

All clones are positive by NcoI digests but XbaI site seems to be missing (either from vector or from insert)

C. Cho

test of Flp expression in pACYC-RIL/BL21DE3 cells

picked colonies from transformations on page 93 (one each expression clone in each RIL strain) and inoculated o/n cultures (1 ml Amp/Cam LB)

Induction of Flp expression in BL21DE3/RIL strains

inoculated 1 ml LB (no antibiotic) with 50 µl of o/n culture

↓

grown for 2½ at 37°C in shaker

↓

removed 100 µl culture, added loading buffer and boiled for 2' → uninduced samples

↓

added 2 µl IPTG (500 mM → 1 mM final) to pCalFlp samples

↓

incubated for another 2h at 37°C in shaker

↓

removed 100 µl from culture, added 100 µl SDS loading buffer and boiled for 2' → induced samples

→ left uninduced E.coli Flp grow for another 2½h (induced)

↓

removed 100 µl culture, added 100 µl SDS loading buffer and boiled for 2' → induced samples

New attempt at LIC cloning ~~Flp~~ pACYC-LIC

4 µl LIC-tailed Flp fragment (page 81)
1 µl LIC-tailed pACYC-LIC vector (from Reinhild)
5 µl dd H2O
incubated 1h at RT, extracted 3x with Strataclean → transformed XL1-Blue with 1 µl of LIC reaction → on LB Cam

Detection of Flo expression in pET BL21 DE3 RIC strains

loaded & Novex 4-20% gels each with pCal Flo, induced/uninduced in RIC 49, 50 and 55 (only induced) and E.b Flo induced/uninduced in RIC 49 and 55

↓

ran gels 1.5h at 150V

↓

one gel stained with Coomassie

other gel transferred to Nitrocellulose membrane by semi-dry transfer (Novex apparatus) for 2h at 30mA

Western blotting of Flo clones with new anti CBP antibody from antibody production group.

blocked membrane with 5% dry milk in TBS for 1h

↓

incubated membrane with 10ml TBS/5% dry milk rabbit anti CBP polyclonal 1:1000 dilution for 1h

↓

washed 4x 5' with TBST

↓

incubated 1h with 10ml HRP-conjugated anti rabbit AB in TBS/5% dry milk

↓

washed 3x 5' with TBST
1x 5' with TBS

developed with Pierce Supersignal system

signal extremely strong. Had to add a piece of paper between the membrane and the film to achieve reasonable exposure times (chemoluminogram next page)
took image with Eagle Eye (signal strong enough for that)

C. Otto